Eyeblink Conditioning in the Infant Rat: An Animal Model of Learning in Developmental Neurotoxicology

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Classical conditioning of the eyeblink reflex is a relatively simple procedure for studying associative learning that was first developed for use with human subjects more than half a century ago. The use of this procedure in laboratory animals by psychologists and neuroscientists over the past 30 years has produced a powerful animal model for studying the behavioral and biological mechanisms of learning. As a result, eyeblink conditioning is beginning to be pursued as a very promising model for predicting and understanding human learning and memory disorders. Among the many advantages of this procedure are (a) the fact that it can be carried out in the same manner in both humans and laboratory animals; (b) the many ways in which it permits one to characterize changes in learning at the behavioral level; (c) the readiness with which hypotheses regarding the neurological basis of behavioral disorders can be formulated and tested; (d) the fact that it can be used in the same way across the life-span; and (e) its ability to distinguish, from normative groups, populations suffering from neurological conditions associated with impaired learning and memory, including those produced by exposure to neurotoxicants. In this article, we argue that these properties of eyeblink conditioning make it an excellent model system for studying early impairments of learning and memory in developmental neurotoxicology. We also review progress that has been made in our laboratory in developing a rodent model of infant eyeblink conditioning for this purpose. — Environ Health Perspect 102(Suppl 2):131–139 (1994).

Key words: developmental neurotoxicology, learning disorders, eyeblink conditioning, cerebellum, hippocampus

Introduction

The prevalence of developmental learning disabilites in our society has generated much concern over the need for research that can provide early assessment and treatment, as well as identify the fundamental causes, of developmental learning disorders (1,2). Although many factors can contribute to the etiology of these disorders, it is becoming increasingly clear that exposure to chemicals during the prenatal and early postnatal period is an important one. It is well established that developmental exposure to drugs of abuse and certain environmental chemicals can adversely affect the development of brain and behavior in humans and laboratory animals (3,4). The cases of environmental lead and fetal alcohol syndrome are two widely known examples that have received much coverage in the popular media (5,6). Mental retardation produced by the developmental neurotoxicity of these and other chemicals has motivated the development of animal models for studying impaired neurocognitive development (7). In addition to experimentally confirming relationships that are commonly correlational in human studies and identifying potential biological mechanisms of these relationships; they also provide data for safety evaluation of chemicals that can inform human risk assessment in the absense of actual human exposure (4).

Risk assessment is a formal process used by the government and private industry to assess the safety of chemicals under certain exposure conditions. Although human toxicity data are used when possible, animal models play a vital role in this process (8–10). Currently, risk assessment in neurotoxicology makes use of the "safety factors approach," in which the level of exposure that is without adverse effect (the no-adverse-effects-level, NOAEL) in an empirical study is divided by a number of safety, or uncertainty factors [UFs, (11)] to derive a reference dose (RfD), the exposure level thought to pose little or no health risks to humans (9,10). Safety factors are used to allow for uncertainties in the empirical data surrounding variables that may determine sensitivity to a neurotoxicant, variables such as differences in the species or age of test subjects, or in route or duration of exposure.

The limitations of the safety factors approach have generated much interest in the environmental health sciences in developing alternative approaches to risk assessment that incorporate information about the biological mechanisms of toxicity (8,12). For example, biologically based models seek to develop mechanistic explanations of toxicity that address species differences in a manner that would reduce or supplant the need for uncertainty factors (12). Such models consider both the relationship between exposure to a compound and the dose delivered to target tissues (exposure-dose) and the relationship between this dose and its subsequent toxic effects (dose-effect). A quantitative model that covers this exposure-dose-effect relationship has recently been developed for studying chemical-induced carcinogenicity (12). Predicting human cancer risk posed by exposure to a given chemical is achieved by applying appropriate values for humans

to the parameters of this model. To our knowledge, no models of this kind have yet been developed to predict learning and memory disorders in developmental neurotoxicology.

To develop a biologically based model of neurotoxicant-induced memory impairment, the effects of chemical exposure on memory must be examined in both humans and animals in terms of intervening effects on the nervous system (Figure 1). The relationship between exposure and tissue dose (neurotoxicant and brain) is addressed by physiologically based pharmacokinetic (PBPK) models, whereas the relation between tissue dose and neurotoxic effect (brain and memory) is addressed by biologically based dose-response (BBDR) models. To accomplish this, one must study a form of memory that *a*) allows one to characterize changes in learning at the behavioral level in a number of ways; *b*) is well understood at the neurobiological level; *c*) lends itself readily to parallel studies in both humans and laboratory animals; *d*) can be used to study the same form of learning in developing organisms (and across the life-span) in a manner that provides a link with maturation of relevant neural systems; *e*)and is able to detect the effects of developmental neurotoxicants that interfere with maturation of brain systems that are involved in memory.

The thesis of the present article is that classical conditioning of the eyeblink reflex offers the potential to be a valuable model for this purpose. It has been argued elsewhere that eyeblink conditioning represents a powerful approach to the study of neurotoxicant-induced neurodegenerative disorders that are associated with memory loss during aging (*13,14*). We will argue that this learning preparation is equally powerful as an animal model for studying developmental disorders of learning and memory and describe progress that our laboratory has made toward establishing infant eyeblink conditioning as a model of learning in developmental neurotoxicology.

Advantages of Eyeblink Conditioning for the Study of Developmental Learning Disorders

In this section we will describe the advantages of using Pavlovian conditioning of the eyeblink reflex to study developmental learning disorders. Few behavioral testing procedures lend themselves to behavioral analysis, neurobiological analysis, comparisons across species (animal-to-human), comparisons across the life-span, and to studies of abnormal brain development as well as eyeblink conditioning does
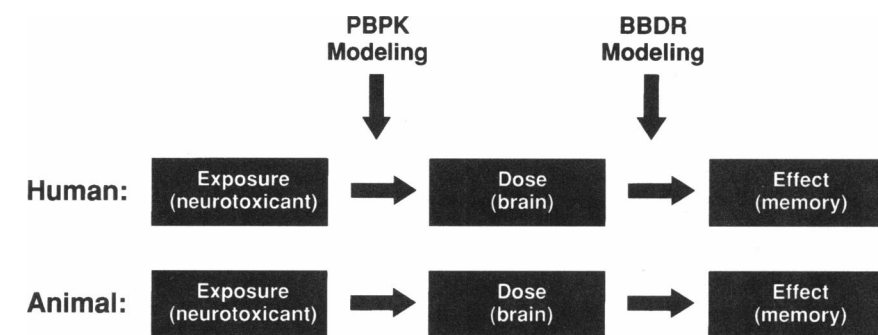

Figure 1. A schematic diagram representing the elements of a biologically based model of memory disorders in neurotoxicology.

(or has the potential to do). Some of these advantages have been discussed by others in connection with studies of the neurobiology of learning (*15,16*) or of learning in human populations suffering from aging-related neurodegenerative disorders (*13,14*). In addition to these, we will argue that the eyeblink conditioning paradigm offers some special advantages that apply more uniquely to the analysis of developmental disorders.

Behavioral Characterization

Eyeblink conditioning is a Pavlovian conditioning procedure that involves contingent, temporal pairings of a conditional stimulus (CS, typically a pure tone) and an unconditional stimulus (US, typically a brief airpuff to the eye). The airpuff elicits a reflexive eyeblink and, following repeated conditioning trials, this response comes to be evoked by the tone CS prior to or in the absense of the airpuff US. This simple form of associative learning has been characterized extensively at the behavioral level (*17*) and possesses several features that aid behavioral analysis of learning and memory. These include its ability to distinguish associative versus nonassociative sources of behavioral change, address the issue of learning versus performance, permit variation in a range of parameters that are important for associative learning, and provide a family of learning phenomena that vary in complexity and in the number of "higher order" neuropsychological processes that are engaged.

The associative nature of eyeblink conditioning is established by comparing performance of subjects trained with contingent, temporal pairings of tone and airpuff with that of control subjects that receive tone and airpuff presented in an unpaired, or randomly paired manner. The very low percentage of eyeblink responses to the tone in such control subjects indicates that eyeblink conditioning is an asso-

ciative phenomenon and that sensitization or pseudoconditioning contribute relatively little to performance (*18*).

Another useful feature of eyeblink conditioning is that the integrity of the blink reflex to the airpuff itself (the unconditioned response, UR) is readily distinguished from learned responses to the tone which precede airpuff onset (the conditioned response, CR). Thus, the possibility that a neurotoxicant, a neurological condition, or some other behavioral or biological variable has influenced the conditioned response (learning) or the reflex itself (performance) can be monitored easily on a trial-by-trial basis.

One can further isolate the effects of such variables on neuropsychological function in this procedure by manipulating a number of parameters. Variations in intensity or duration of the tone or airpuff, the interval between tone and airpuff onset, or other aspects of the temporal or contingent relationship between these stimuli, can be used to assess the role of sensory, motor, and motivational processes in learned performance. Such variations also produce characteristic effects on eyeblink conditioning (*17*). The fact that these "laws of learning" appear to apply similarly across mammalian species (including humans) suggests that similar neural and psychological mechanisms are engaged (*14,19*).

Finally, there are a number of "higher order" phenomena of eyeblink conditioning that make it is possible to study the manner in which processes of attention (*20*), memory (*21*), and perceptual organization (*22*) modulate associative learning; and to assess the integrity of brain systems involved in these processes [(*23*); see below] For example, delay and trace conditioning have been contrasted in studies of memory loss associated with aging. In delay conditioning, tone and airpuff overlap in time, whereas in trace conditioning, tone and airpuff are temporally separated by an

appropriate "trace interval." Aged rabbits show deficits in trace but not delay conditioning (*13*).

In summary, there are many important behavioral features and properties of eyeblink conditioning that greatly enhance the sophistication with which one can characterize learning impairments associated with abnormal development.

Neural Basis of Eyeblink Conditioning

A perhaps more significant advantage of eyeblink conditioning, is the impressive degree to which the neural circuitry supporting this form of learning has been studied and characterized. A model of the essential neural circuitry for eyeblink conditioning has been proposed that includes elements of cerebellar and brain stem systems (Figure 2) (*24*). In this model, neural activity representing the airpuff unconditioned stimulus (US) is transmitted to the cerebellum via climbing fibers that arise from the dorsal accessory olive (DAO). Electrical stimulation of the DAO can serve as an effective US when paired with the tone conditioned stimulus (CS) (*25*). Furthermore, when the tone and DAO stimulation are explicitly unpaired, rapid extinction of the CR occurs. Neural activity representing the conditioned stimulus arises from the pontine nuclei and enters the cerebellum via the mossy fiber projection. Electrical stimulation of the mossy fiber input to the cerebellum can serve as a
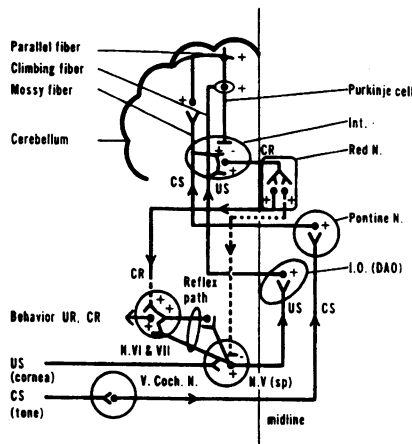

Figure 2. Schematic diagram depicting the hypothesized circuitry for eyeblink conditioning. The CS pathway enters the cerebellum via projections from the pontine nuclei (Pontine N). The US pathway enters the cerebellum via projections from the inferior olive (I.O.). The CS and US pathways converge on the nucleus interpositus (Int.). The CR pathway leaves the Int., synapses with the red nucleus (Red N), and ultimately descends to motor neurons that control the eyeblink reflex. From Thompson (*24*); reprinted with permission.

CS when it is paired with an airpuff US (*26*). Thompson (*24*) has hypothesized that the site of the memory trace for this conditioned response is at the point where mossy fiber inputs (CS) and climbing fiber inputs (US) converge, the cerebellar deep nuclei and cortex. This notion is supported by the finding that lesions of the cerebellar deep nuclei completely and irreversibly abolish conditioned responding (*27–30*). The conditioned response pathway extends from the ipsilateral interpositus nucleus, crosses the midline, synapsing in the magnocellular region of the red nucleus. The red nucleus projects to motor neurons that control the eyelid response. This model accounts for most of the data concerning the neural circuitry required for delay conditioning in eyeblink conditioning. To be able to specify the neural substrates of learning at this level of detail creates an almost unique opportunity to use eyeblink conditioning for studies that can benefit from understanding brain-behavior relations.

Although it is clear that the "essential" circuitry for eyeblink conditioning is below the level of the thalamus (*24,31*), hippocampal activity during acquisition can also modulate the rate of learning (*32*). Berger and colleagues have found that hippocampal neuronal activity forms a perfect model of the behavioral changes during nictitating membrane conditioning in the rabbit (*33*). The pattern of CS-evoked pyramidal cell activity directly models the shape of the conditioned response (*34*). Moreover, manipulations that alter hippocampal electroencephalogram (EEG), including medial septal lesions and the application of Δ-9-tetrahydrocannabinol (THC), retard the rate of acquisition (*32*). Cholinergic blockade also retards the rate of eyeblink conditioning (*35–37*). Thus, although hippocampal lesions do not impair eyeblink conditioning (*38*), acquisition rate is modulated by hippocampal activity.

In addition to modulating rate of delay conditioning, there is also extensive evidence for hippocampal involvement in higher order phenomena of eyeblink conditioning. Examples of such phenomena include trace conditioning (*21*), latent inhibition (*20*), discrimination reversal (*39*), and sensory preconditioning (*40*). Thus, it appears that eyeblink conditioning is mediated by an interaction between the hippocampus (and associated forebrain structures) and brainstem cerebellar pathways. Indeed, a mathematical model describing how this interaction may sub-

serve different phenomena of eyeblink conditioning has recently been formulated (*23*). This model makes it possible to make quantitative predictions regarding the effects of damage to hippocampal-cerebellar pathways on various behavioral phenomena of eyeblink conditioning. In the context of risk assessment in developmental neurotoxicology, this model has the potential to serve as a BBDR model relating nervous system toxicity to memory impairment (Figure 1).

In summary, there has been extensive empirical and theoretical progress in the analysis of the neurobiological mechanisms of eyeblink conditioning. As a result, this learning preparation offers unusual potential to understand the behavioral effects of developmental neurotoxicants in terms of their potential neural target sites of action (see below).

Comparison of Humans and Laboratory Animals

Another advantage of eyeblink conditioning is that it makes it possible to study learning in humans and in laboratory animals with the identical procedure (*13,14*). Preparations for studying eyeblink conditioning exist in rats (*30,41,42*); rabbits (*16,17*), and humans (*14*). Moreover, the general behavioral properties of eyeblink conditioning appear to be similar across species. Perhaps more importantly, the evidence that is available thus far suggests that the biological mechanisms of eyeblink conditioning are similar across species as well. Comparisons of studies involving humans and animal models indicate that the effects of a number of biological variables on eyeblink conditioning are similar in different species. For example, cerebellar damage, hippocampal damage, aging, and administration of anticholinergic drugs are variables that all appear to have similar effects in humans and laboratory animals (*14*). The fact that the behavioral and biological properties of eyeblink conditioning appear to have been conserved across mammalian species could significantly increase the ability of animal studies involving this procedure to characterize and predict neurotoxicity in humans.

Comparisons Across the Life-Span

In addition to cross-species comparisons, the eyeblink conditioning procedure is almost unique in the degree to which it lends itself to comparisons across the life-span (*43*). Because eyeblink conditioning does not require language competence and makes relatively simple sensorimotor

demands on test subjects, the identical test procedure can be used in neonatal human infants as young as 10 to 20 days of age (44) or in senior citizens older than 80 years of age (45). Studies of eyeblink conditioning have also been carried out in aged rats (42) and rabbits (13,43). In fact, normal aging produces a decline in the rate of eyeblink conditioning in both human and animal subjects (in a certain proportion of the test population). It has been hypothesized that this decline reflects aging-related changes in the neural systems that are involved in eyeblink conditioning (13,14). It has also been reported that patients with Alzheimer's disease show even more pronounced deficits in eyeblink conditioning relative to age-matched controls. There is evidence that aluminum neurotoxicity may be a risk factor for this neurological disorder and studies that have used aluminum-exposed rabbits as a model of Alzheimer's disease have demonstrated acquisition and retention deficits with the eyeblink conditioning paradigm (13,46,47).

Applications to Developmental Learning Disorders

The success of eyeblink conditioning as an approach to the study of aging-related memory disorders is encouraging and suggests that it would also be fruitful for the study of developmental learning disabilities. The neural systems that are important for eyeblink conditioning—the cerebellum and hippocampus—undergo protracted postnatal development in humans and other animals (48,49). This protracted development makes these systems likely targets of developmental neurotoxicants and there are studies suggesting that this is indeed the case for a number of chemical compounds. There is also evidence that certain developmental learning disorders are associated with neuropathology in these regions. Finally, the fact that the procedure can be carried out in human infants creates an unusual opportunity to perform parallel studies in humans and in laboratory animals. For these reasons, we have undertaken an effort to develop a preparation for studying eyeblink conditioning in infant rats. In this section, we will briefly elaborate some of these points. We will then describe our progress in implementing an animal model for studying the early development of eyeblink conditioning.

The neuroanatomical structure that appears to be critical for eyeblink conditioning, the cerebellum (Figure 2), undergoes massive and prolonged postnatal maturation. In the rat, the volume of the

cerebellar cortex increases more than 20-fold during the first three postnatal weeks (Figure 3) (50). Cells of the deep nuclei, and Purkinje cells are formed before birth, but granule, stellate, and basket cells are generated and proliferate after birth. Most stellate and basket cells are formed during the first two weeks after birth. Granule cells are generated throughout the first three postnatal weeks with a peak in proliferation during the third week (Figure 3) (49). In addition to the massive generation of microneurons in the cerebellar cortex, each of the cell types within this structure undergoes extensive morphological development and synaptogenesis during the first few postnatal weeks (49). The late development of the cerebel-

lum may make it vulnerable to some developmental neurotoxicants. Indeed, certain solvents (ethanol and methanol), heavy metals (e.g., methylmercury) and antimitotic agents have been reported to disrupt cerebellar development (51). As a result, eyeblink conditioning could provide a very powerful means of assessing learning deficits associated with developmental exposure to these neurotoxicants. The hippocampus and related structures also undergo substantial postnatal growth and development in a variety of mammalian species (48,52–54). For example, studies of the rat have shown that 72% of dentate granule cells are generated between birth and 16 days of age, and 94% of granule cell
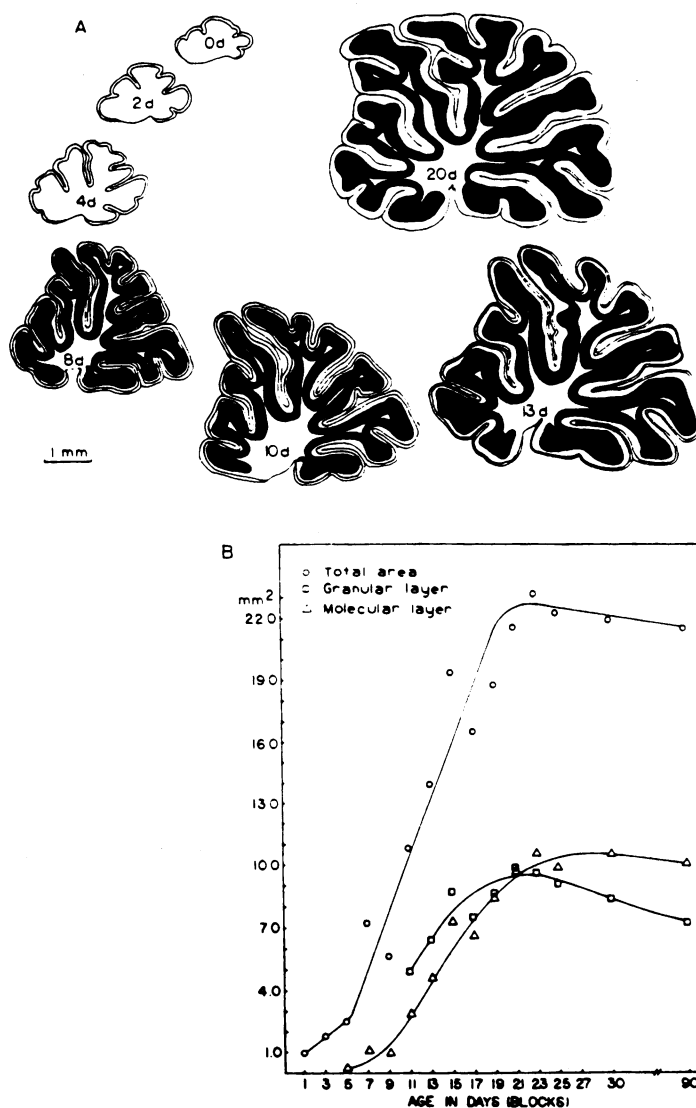

Figure 3. A Sagittal view of the vermis of rats of different postnatal ages. B. Graph of areal and laminar postnatal growth of the cerebellar cortex. From Altman (49); reprinted with permission.

synapses appear between 11 and 25 days of age. Additionally, levels of hippocampal acetylcholinesterase activity and myelin staining show substantial postnatal increases (48). It is also well established that hippocampal development can be altered by a range of developmental insults (55). These include antiproliferative agents (48,56); ethanol (57-59); and heavy metals (7,60). Because of the role of the hippocampal formation in certain phenomena of eyeblink conditioning , this preparation may also be useful for assessing impairments in memory development that are produced by developmental damage to this forebrain structure.

There is also evidence that certain disorders of human behavioral development, such as those associated with autism, mental retardation, undernutrition, and developmental methylmercury neurotoxicity are sometimes accompanied by cerebellar damage or hypoplasia (51,61-63). There are also many human neurological disorders associated with pathology to the hippocampus and/or related structures, including ischemia (64), Down's syndrome (65), schizophrenia (66), undernutrition (67), hypoglycemia (68), hypothyroidism (69), early exposure to lead (70,71), and fetal alcohol exposure (72,73). As a result, eyeblink conditioning could be useful in the early detection and characterization of functional loss associated with these neurological conditions.

This possibility is supported in a study by Ohlrich and Ross (74), in which normal and mentally retarded children were trained with delay conditioning, discrimination, and discrimination reversal procedures with the eyeblink conditioning preparation. Normal children showed higher levels of conditioning with an 800 msec interstimulus interval (ISI) than a 500 msec ISI and showed discrimination learning. Mentally retarded children did not benefit from the ISI manipulation and did not show discrimination learning, although they did show moderate levels of conditioning at both 500 and 800 msec ISIs. Another study examined college-aged normal and mentally retarded subjects with delay conditioning and partially reinforced groups (75). The mentally retarded subjects were impaired on acquisition and extinction of eyeblink conditioning. The ability of eyeblink conditioning to distinguish normal and mentally retarded children and adults suggests that this procedure may be useful for assessing and characterizing a variety of human neurological conditions associated with impaired

intellectual development. Because of the involvement of the cerebellum and hippocampus in eyeblink conditioning, this prospect seems particularly likely for neurological disorders associated with neuropathology or impaired maturation of these structures. When such disorders are produced by developmental exposure to drugs and chemicals, it would be possible to carry out parallel studies in animal models. Our work with an animal model of infant eyeblink conditioning is described in the next section.

A Rodent Model for Studying the Development of Eyeblink Conditioning

In this section, we describe efforts in our laboratory to develop a rodent model for studying the early development of eyeblink conditioning. Thus far, our studies have asked whether there are ontogenetic changes in eyeblink conditioning in the rat, whether such changes reflect maturation of associative processes, and whether neurotoxicants that interfere with cerebellar maturation impair eyeblink conditioning during infancy. Because some of these experiments have not been extensively reported elsewhere, we will describe certain aspects of our methods and procedures in some detail.

Eyeblink Conditioning Is Associative and Develops Postnatally in the Rat

The developmental analysis of eyeblink conditioning began when a method for conditioning freely moving adult rats (30) was adapted for use with the infant rat (76). In this procedure, rats are surgically implanted with two electrodes, one for measuring eyelid electromyographic (EMG) activity and one for delivering brief electrical stimulation in the vicinity of the

eye. Subjects are placed in an enclosure containing a speaker for delivering the auditory CS; electrodes are connected via a headstage and commutator to peripheral devices and a personal computer which collects data and controls experimental events. In the first study that we will describe, rat pups were trained in eyeblink conditioning on postnatal day (PND) 17 or 24. At each age, pups were trained with delay conditioning or unpaired control procedures. Delay conditioning trials involved pairings of a 380 msec tone conditioned stimulus (CS) and a 100 msec perioccular-shock unconditioned stimulus (US). In the unpaired control condition, the CS and US were presented in a "pseudorandom" order such that no more than three presentations of either stimulus occurred consecutively. The paired and unpaired groups received the same number of stimulus presentations at the same average rate across each session. This unpaired group is an important control because it indicates levels of sensitization, pseudoconditioning, and/or spontaneous EMG activity which could lead one to overestimate the amount of associative learning in the paired condition.

The results of this experiment are shown in Figure 4. Rats trained on PND 17 in the paired condition (left panel; closed circles) showed much less conditioning over 300 trials than rats tested on PND 24 (right panel; closed circles). Moreover, the difference in rate of conditioning reflected a difference in the amount of associative learning, as indicated by the difference between given paired and unpaired groups at each age. Pups trained on PND 24 showed a greater difference between paired and unpaired conditions (right panel; closed vs open circles) than pups trained on PND 17 (left panel; closed vs open circles). These results clearly demon-
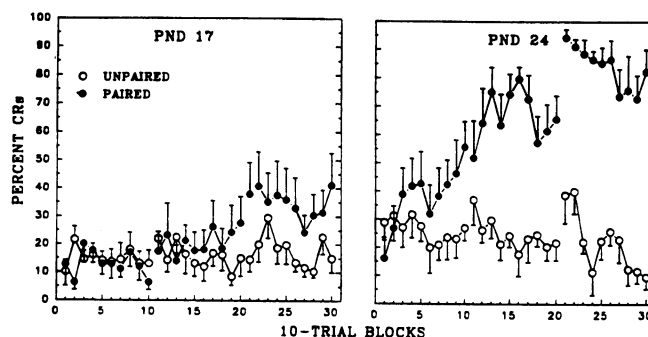

Figure 4. Results of an experiments designed to test the development of associative eyeblink conditioning in 17-day-old (left panel) and 24-day-old rats (right panel). Data points depict mean (± SEM) percentage of conditioned responses (CRs) as a function of training condition (closed symbols, paired; open symbols, unpaired controls), sessions, and trial blocks (10 blocks of 10 trials per session). Interruptions in the lines connecting data points represent session breaks. From Stanton et al. (76).

strate that associative eyeblink conditioning is possible in infant rats and that rate of conditioning develops dramatically between PND 17 and 24.

Measures of Learning and Performance

As indicated previously, one of the more valuable features of the eyeblink conditioning procedure is that it simultaneously provides measures of learning and measures of performance. The importance of addressing the learning versus performance distinction can be illustrated with these developmental data. One question that could arise from the experiment just described is whether the age-related difference in learned responding was actually the result of differences in the performance capabilities of the younger pups. For example, one could imagine that the younger pups simply could not hear the tone CS or could not perceive or respond to the US as well as the 24-day-old rats. Startle responses can be used to determine whether an animal can hear the tone CS even in the absense of any conditioning. Startles are whole body responses to the tone CS that occur during the first 80 msec of the CS period. Differences in US intensity thresholds or in amplitude of the unconditioned eyeblink response provide information about the ability of pups to generate a defensive blink response per se. This unconditioned response can be distinguished operationally from responses to the tone prior to, or in the absence of, the US, which provides information about the pup's ability to demonstrate learning.

Figure 5 shows an analysis of US intensity thresholds and unconditioned response amplitudes, and the percentage of startle responses in pups trained as weanlings or preweanlings. Preweanling pups showed at least as many startle responses to the tone CS as weanling pups (left panel, the two age groups did not differ statistically). Furthermore, when the US level was the same for both groups, preweanling pups produced unconditioned responses that were the same amplitude as those produced by the weanling pups (right panel). These results indicate that the difference in the rate of conditioning was not owing to the inability of preweanling rats to perceive or respond to the US or to their inability to hear the CS.

Disrupting Cerebellar Development Impairs Eyeblink Conditioning in the Infant Rat

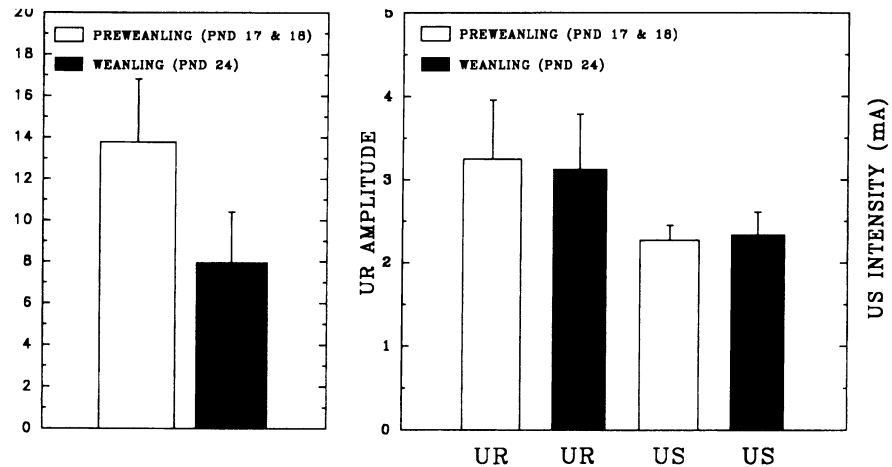Various antiproliferative agents given during development can cause neuronal and

Figure 5. Left panel: mean (±SEM) percentage startle responses (SRs) as a function of age of testing. SRs were defined as short-latency (<80 msec) eyeblinks to the conditioned stimulus (CS) and were averaged across all training sessions. The trend toward an age difference in this measure of unlearned responding to the CS was not statistically significant. Right panel: mean (±SEM) unconditioned response (UR) amplitudes (left scale) and unconditioned stimulus (US) intensities (right scale) used as a function of age of testing. US intensities were determined separately for each individual subject, but the average intensity employed did not differ across age (21).

behavioral deficits in rodents that vary according to age of exposure (77). For example, early postnatal exposure to X-irradiation (49) or methylazoxymethanol (MAM; 78,79) produces massive deficits in the development of cerebellar cortical neurons and deficits in the development of motor skills. The development of different cell types within the cerebellar cortex can be reduced by exposure to MAM, depending on the timing of exposure and neuronal birth dates (78–80). For example, most cerebellar granule cells are generated postnatally (81) and MAM exposure on the day of birth produces a large reduction in cerebellar granule cells, but not Purkinje cells (79). Thus, the late development of microneurons in the cerebellar cortex make its circuitry especially susceptible to neurotoxicants during postnatal development.

We recently conducted an experiment that was designed to determine whether disrupting the development of the cerebellum by neonatal exposure to MAM would
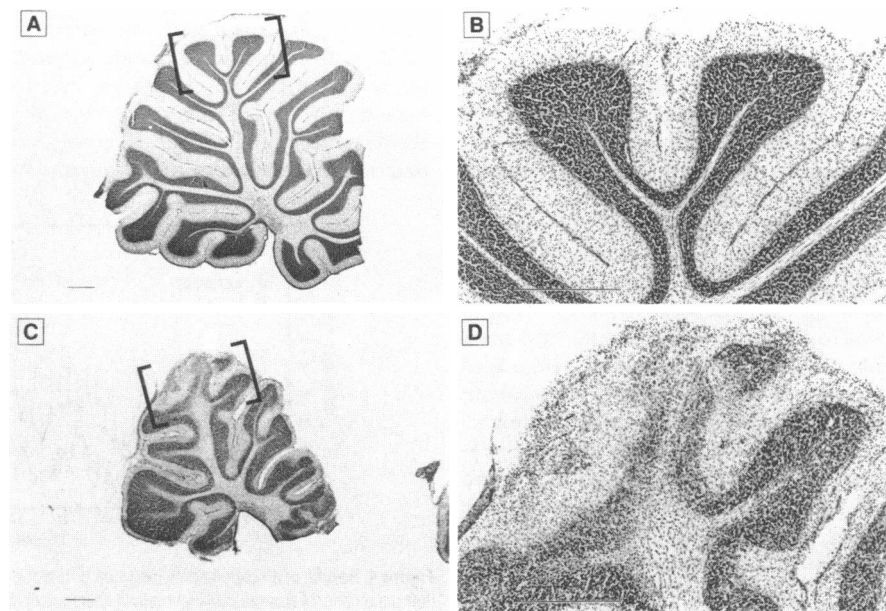

Figure 6. Sagittal cerebellar sections from 25-day-old rats injected with saline (A,B) or MAM [([20 mg/kg]) C,D] on PND 0–1. Cerebellar hypoplasia was clearly evident in MAM-treated rat pups.
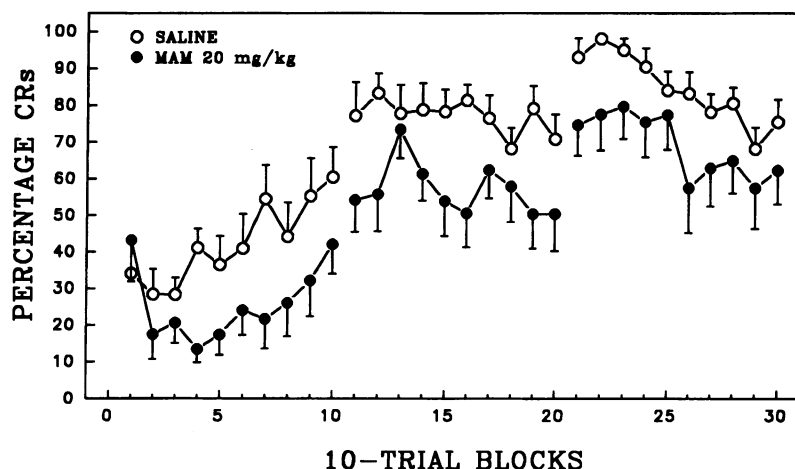
Figure 7. Data from an experiment examining the effects of MAM on eyeblink conditioning in infant rats. Data points represent mean (±SEM) percentage conditioned responses (CRs) for rats given 20 mg/kg MAM (filled circles) or saline (open circles) as a function of sessions, and trial blocks (10 blocks of 10 trials per session). Interruptions in the lines connecting data points represent session breaks.

disrupt eyeblink conditioning during infancy (*82*). Rat pups were given two subcutaneous injections of 20 mg/kg MAM, one each on PND 0 and 1, whereas pups in a control group were given two successive injections of saline vehicle. Following MAM exposure, pups were left undisturbed except for periodic cage changes until the beginning of experimental procedures. On PND 24 or 25, pups received three 100-trial sessions of delay conditioning (as described earlier). Because pups in this study were trained in a different apparatus than was used in our previous reports (*76,83*) we will describe it briefly. The modified conditioning apparatus consisted of four small animal chambers, each lined with sound absorbing foam and each housing a small stainless steel mesh cage where the animal was placed during conditioning. One wall of the chamber was fitted with two audio speakers that could independently produce tones of different frequencies. In other respects, the apparatus was similar to that used previously (*30,76,83*).

Figure 6 shows the effects of neonatal exposure to MAM on the cerebellum in sagittal sections. Comparison of sections from rats injected with saline (A,B) or MAM ([20 mg/kg] C,D]) show that cerebellar hypoplasia was clearly evident in MAM-treated rat pups. Further, the cellular structure of the cerebellar cortex was clearly disrupted in MAM treated pups as demonstrated by the dispersion of Purkinje cells throughout the granule cell layer in some cerebellar lobules (Figure 3D).

The behavioral results are shown in Figure 7 in terms of the percentage of conditioned responses per 100-trial training session. Pups neonatally exposed to MAM

(closed circles) showed much less conditioning than pups given saline [open circles; (*p*< 0.05)], although both groups showed an increase in conditioning across sessions (*p*< 0.05). Thus, neonatal exposure to MAM produced cerebellar hypoplasia and a deficit in eyeblink conditioning in weanling rats.

This finding supports the notion that developmental neurotoxicants that disrupt maturation of the cerebellum will also disrupt the ontogeny of eyeblink conditioning, a form of associative learning that, in adult animals at least, critically involves this brain structure (*27*). This conclusion, of course, depends on the assumption that MAM did not significantly disrupt maturation of other brain systems. At the age of exposure (PND 0–1) employed in this experiment, the effects of MAM on the cerebellum are much more striking than effects on other gross neuroanatomical regions (*79*). For example, the neocortical hypoplasia that is seen with gestational day 15 exposure to MAM (*80*) is not readily apparent following exposure on PND 0-1. Nevertheless, it is possible that MAM may have affected neurons outside the cerebellum (e.g., granule cells in hippocampus or olfactory bulb) that proliferate during this period of development. To assess possible effects of MAM on forebrain structures, the same rats that were tested for eyeblink conditioning were also tested on delayed alternation in a T-maze under conditions that reveal clear effects of neonatal damage to the septo-hippocampal pathway or prefrontal cortex (*84,85*). There were no effects of MAM treatment on acquisition of this alternation task (*82*). Thus, the learning impairment produced by exposure to MAM on PND

0–1 was not a general one but rather appeared only on the task, eyeblink conditioning, for which the cerebellum is thought to be critical.

In summary, we have shown that eyeblink conditioning procedures can be carried out in infant rats and that this form of conditioning emerges between 17 and 24 days after birth. The failure of 17-day-old rats to show conditioning does not reflect sensory or motor impairment because startle responses to the auditory CS and unconditioned eyeblink responses to the US do not differ from those observed in 24-day-old rats. Finally, disrupting cerebellar maturation with perinatal MAM exposure impairs acquisition of the conditioned eyeblink response during postnatal development without disrupting other forms of learning that involve forebrain structures, but for which the cerebellum is apparently less critical. These findings suggest that eyeblink conditioning in the developing rat has significant potential as an animal model of learning in developmental neurotoxicology. Additional research to further develop and elaborate this model is clearly warranted.

Summary and Conclusions

In this article, we have argued that eyeblink conditioning promises to provide a powerful paradigm for studying developmental learning disorders associated with exposure to neurotoxic chemicals. This procedure makes it possible to study associative learning in the same manner in both humans and laboratory animals, and throughout the lifespan. The neurobiological mechanisms of this form of learning are becoming well characterized and there is evidence that these mechanisms are similar in humans and laboratory animals. Eyeblink conditioning distinguishes normal individuals from those suffering from neurological conditions that impair learning and memory. Because the cerebellum plays an essential role in generating the conditioned eyeblink response, this learning paradigm could be useful for examining learning deficits in infancy that are associated with cerebellar pathology. In addition, there are phenomena of eyeblink conditioning that may reflect damage to the hippocampus and related structures. We have performed preliminary studies with the antiproliferative agent, MAM, which suggest that disrupting cerebellar maturation impairs eyeblink conditioning in infant rats. Further use of this paradigm in developing animals and humans should both increase our understanding of the fundamental mechanisms of developmental learning disorders as well as improve our ability to predict these disorders in humans on the basis of chemical safety evaluations performed in animals.

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